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Note

High-performance liquid chromatographic method for the analysis of isoflavones

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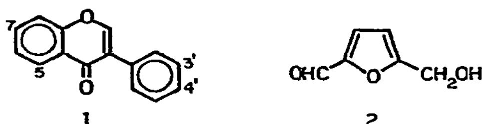
The isoflavones (1) are key intermediates in isoflavonoid biosynthesis[1]. They are largely restricted to the sub-family Lotoideae of the Leguminosae[1,2]. However, because this family contains many economically important and otherwise interesting plants and because the isoflavones possess oestrogenic and fungitoxic properties, they have been included in many biosynthetic, phytopathological and taxonomic studies[1,2].



One result of these studies has been the development of a variety of chromatographic methods for the analysis of isoflavones in plant tissues. These methods rely predominantly on thin-layer (TLC) or column chromatographic techniques[3]. Paper and gas chromatography have found occasional use in the separation of isoflavones[3]. However, the poor resolution observed for the former procedure and the need for derivatization and the possibility of artifact formation in the latter case do not make these methods particularly attractive[4]. Although TLC and related procedures have usually been the methods of choice for isoflavone analysis the available references to their use illustrate that they frequently do not provide sufficient resolution. As a result, the analysis of plant tissue isoflavone content can require the application of multiple chromatographic steps to ensure isolate homogeneity.

The ability of high-performance liquid chromatography (HPLC) to resolve multicomponent mixtures for quantitation or component isolation has been well established. However, with the exception of a recent report of isoflavone HPLC on a reversed-phase column[5] the use of HPLC for isoflavone resolution has not been investigated. Unfortunately, the severe band spreading which was observed with the reversed-phase system would make the resolution of multicomponent mixtures virtually impossible.

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This report describes an HPLC method which will satisfactorily resolve isoflavone mixtures. Its use for the separation of isoflavone mixtures which span a range of polarities as well as for the resolution of closely related compounds has been demonstrated. The analysis of isoflavone aglycones and glycosides (as aglycones) has been shown using *Glycine max* (L.) Merr.

MATERIALS AND METHODS

Chromatography

The liquid chromatograph used in this study was a Waters Assoc. ALC 202 equipped with a 2-cm Corasil II guard column, a 25-cm μPorasil column and a UV detector with a 280-nm filter. The solvent flow-rate in all of the chromatograms illustrated was 1.5 ml/min. The solvents used were purified by distillation (methylene dichloride, absolute ethanol and acetic acid) or by absorption of trace aromatics on silica gel from hexanes. The solvent front is indicated by sf. All solvent ratios are by volume.

Preparation of isoflavone standards

The following compounds were obtained by published procedures: 4′,7-dihydroxyisoflavone[6], 4′,7-dimethoxyisoflavone[6],4′, 6-dimethoxy-7-hydroxy-γ-isoflavone[7], 7-(γ,γ-dimethylallyloxy)-4′-methoxyisoflavone[6], 7-hydroxy-4′-methoxyisoflavone[8], 4′-hydroxy-7- methoxyisoflavone[8], 7-hydroxy-3′,4′-methylenedioxyisoflavone[8], 7-methoxy-3′,4′-methylenedioxyisoflavone[9] and 4′,5,7-trihydroxyisoflavone[10].

Preparation of soybean samples

The cultural variety of the soybean seed used in this study was not determined.

Ethanolic extraction of the soybean seed (*ca.* 5 gm.) followed the general procedure of Pueppke and Van Etten[11]. The seeds were soaked in water for 1 h and macerated twice with 20 ml of 95% ethanol. After filtration, two volumes of water were added and the ethanol removed under reduced pressure on a rotary evaporator. The aqueous fraction was acidified with 5% aqueous HCl (*ca.* 2 ml) and extracted twice with equal volumes of ether. The solution was dried over anhydrous sodium sulfate, filtered and the solvent removed on a rotary evaporator. The residue was dissolved in methylene dichloride for analysis.

Hydrolytic extraction of the soybean seed followed the general procedure of Harborne[12]. The samples (*ca.* 5 g) were added to 50 ml of 2 *N* HCl and hydrolyzed for 30 min at 100°C. After cooling and filtration through two layers of cheesecloth the solution was twice extracted with equal volumes of ethyl acetate. If necessary minimal quantities of methanol were added to break troublesome emulsions. The solution was dried over anhydrous sodium sulfate, filtered and the solvent removed on a rotary evaporator. The residue was dissolved in methylene dichloride–ethanol (19:1) for analysis.

Identification of the soybean metabolites

The isoflavone and furaldehyde metabolites of soybean were collected after HPLC separation and identified by comparison of their UV, nuclear magnetic resonance (NMR), mass spectrometry (MS), TLC and HPLC properties with authentic samples.

RESULTS AND DISCUSSION

The chromatogram in Fig. 1 illustrates that a gradient solvent system of hexanes, methylene dichloride, ethanol and acetic acid will satisfactorily resolve an isoflavone mixture which spans a range of polarities. A multiple solvent system of this type was chosen because it would allow for both gross and fine variation in solute retention by alterations in the component solvent ratios.

Figs. 2 and 3 demonstrate that the HPLC system can also be used to separate closely related compounds. The resolution of 7-hydroxy-4'-methoxyisoflavone (Formononetin) from 7-hydroxy-3',4'-methylenedioxyisoflavone (Pseudobaptigenin) is

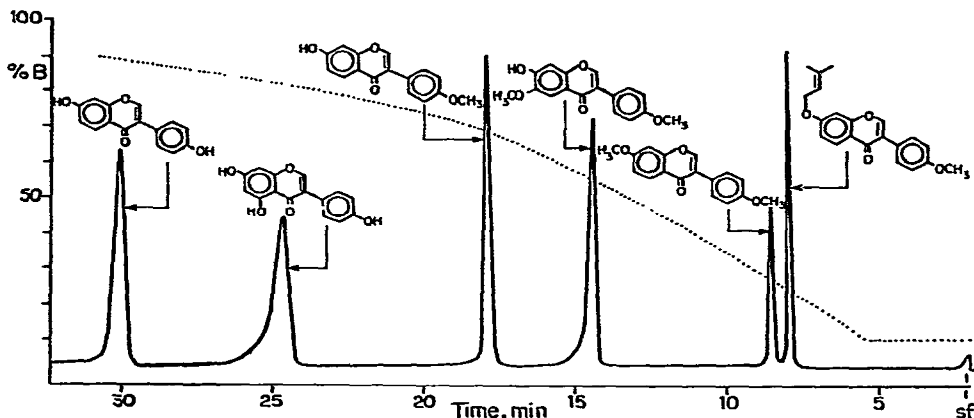

Fig. 1. Gradient chromatogram of selected isoflavones. Solvent A, hexanes; solvent B, methylene dichloride–ethanol–acetic acid (97:3:0.2). The gradient used is shown on the chromatogram.

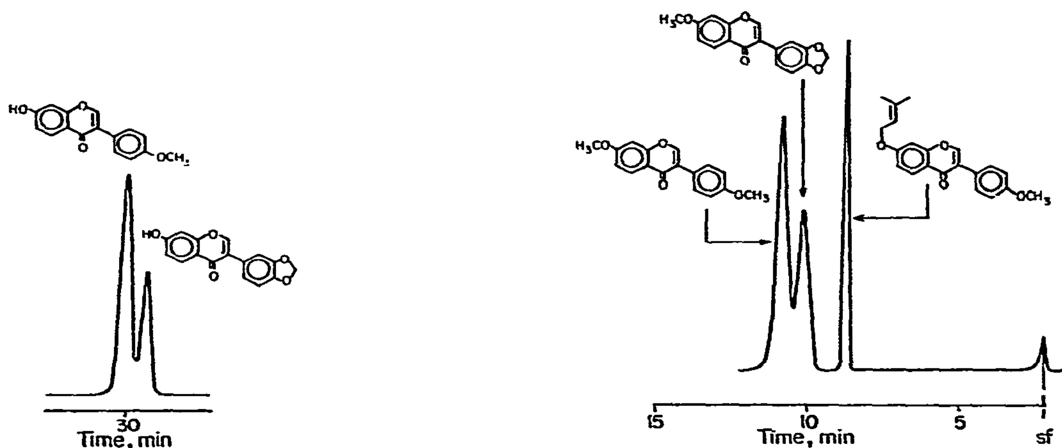

Fig. 2. Gradient chromatogram of 7-hydroxy-4'-methoxy- and 7-hydroxy-3',4'-methylenedioxyisoflavone. Gradient as in Fig. 1 with 0–25 min omitted for clarity. Solvent A, hexanes; solvent B, methylene dichloride–ethanol–acetic acid (99:1:0.2).

Fig. 3. Isocratic chromatogram of 4',7-dimethoxy-, 7-methoxy-3',4'-methylenedioxy- and 7-γ,γ-dimethylallyloxy-4'-methoxyisoflavone. The solvent system was [methylene dichloride–ethanol–acetic acid (97:3:0.2)]–hexanes (12:88).

particularly significant because these compounds have not previously been successfully resolved by a liquid chromatographic procedure[13].

The chromatographic conditions of Fig. 2 were also used in an attempt to separate a mixture of 7-hydroxy-4'-methoxyisoflavone and its 4'-hydroxy-7-methoxyisoflavone isomer. Unfortunately, no resolution was observed.

The application of the HPLC procedure to the determination of isoflavones from plant extracts was tested using *Glycine max* because soybean seed is known to contain daidzein (7,4'-dihydroxyisoflavone) as well as glycosides of daidzein and genistein (5,7,4'-trihydroxyisoflavone)[14].

It is possible to analyze for both isoflavone aglycones and glycosides by the selection of suitable extraction procedures. The aglycones are isolated by alcoholic extraction while the glycosides can be isolated as their aglycones after hydrolysis with aqueous acid[12]. Fig. 4 illustrates that the HPLC method in combination with the ethanolic and hydrolytic extraction procedures does provide a satisfactory method for isoflavone analysis. As was expected, the ethanolic extract contains daidzein while the hydrolytic extract was found to contain daidzein and genistein.

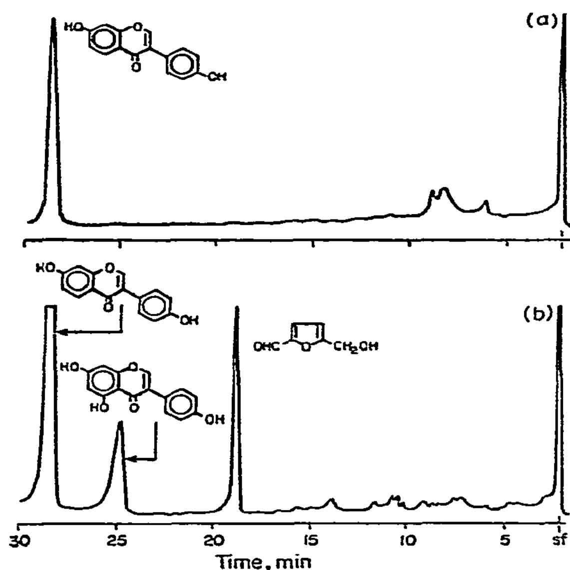

Fig. 4. Gradient chromatograms of the *G. max* extracts. Solvent system and gradient as in Fig. 1. a, Ethanolic extract. b, Hydrolytic extract.

The additional peak in the hydrolytic extract chromatogram was identified as 5-hydroxymethyl-2-furaldehyde (2). This compound has been isolated from a variety of plant products and may be formed by the breakdown of sugars during the hydrolytic extraction[15].

In addition to illustrating the presence of daidzein, genistein and 5-hydroxymethyl-2-furaldehyde the chromatogram of the hydrolytic extract of soybean seed (Fig. 4b) shows that the separation observed for these compounds is greater than that required for acceptable resolution. This suggests that once the composition of a

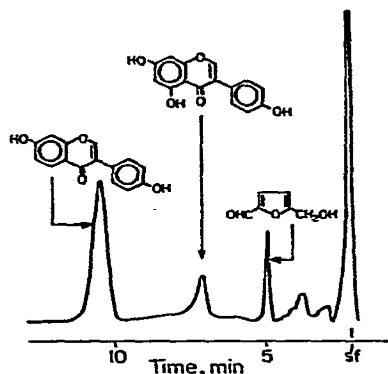

Fig. 5. Isocratic chromatogram of *G. max* hydrolyzate. The solvent system was [methylene dichloride–ethanol–acetic acid (97:3:0.2)]–hexanes (8:2).

sample has been determined using a wide range solvent system which will resolve any unsuspected components (*e.g.* the 5-hydroxymethyl-2-furaldehyde from *G. max*) the chromatographic procedure can be optimized for further studies.

As illustrated in Fig. 5, the same information on daidzein, genistein and the hydroxymethylfuraldehyde can be obtained by the use of an isocratic solvent system. Not only is analysis time reduced from 30 to 10 min but because the isocratic run does not require solvent re-equilibration for each sample the rate of analysis is increased from 1 sample per hour to 5 samples per hour.

ACKNOWLEDGEMENTS

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